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CD4 T-Cell Memory Responses to Viral Infections of Humans Show Pronounced Immunodominance Independent of Duration or Viral Persistence

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Little is known concerning immunodominance within the CD4 T-cell response to viral infections and its persistence into long-term memory. We tested CD4 T-cell reactivity against each viral protein in persons immunized with vaccinia virus (VV), either recently or more than 40 years ago, as a model self-limited viral infection. Similar tests were done with persons with herpes simplex virus 1 (HSV-1) infection as a model chronic infection. We used an indirect method capable of counting the CD4 T cells in blood reactive with each individual viral protein. Each person had a clear CD4 T-cell dominance hierarchy. The top four open reading frames accounted for about 40% of CD4 virus-specific T cells. Early and long-term memory CD4 T-cell responses to vaccinia virus were mathematically indistinguishable for antigen breadth and immunodominance. Despite the chronic intermittent presence of HSV-1 antigen, the CD4 T-cell dominance and diversity patterns for HSV-1 were identical to those observed for vaccinia virus. The immunodominant CD4 T-cell antigens included both long proteins abundantly present in virions and shorter, nonstructural proteins. Limited epitope level and direct *ex vivo* data were also consistent with pronounced CD4 T-cell immunodominance. We conclude that human memory CD4 T-cell responses show a pattern of pronounced immunodominance for both chronic and self-limited viral infections and that this pattern can persist over several decades in the absence of antigen.

Immunodominance refers to the proportion of T cells specific for a defined epitope in relation to the entire set of T cells reacting to a complex antigen (1). For infectious pathogens encoding many polypeptides, the immunodominance of an open reading frame (ORF) is the proportion of the total pathogen-specific response accounted for by T cells reacting with this ORF. Immunoprevalence is a related concept, referring to the proportion of a population responding to an immunogen (2). The CD8 T-cell response to complex microbes can show remarkably strong immunodominance in humans and inbred animals.

As antigen processing differs between T-cell subsets, it is not clear that immunodominance also applies to CD4 T-cell responses. For VV, memory CD4 T-cell responses in inbred mice are quite polyclonal and do not exhibit dominance. The top 14 epitopes account for only 20% of the total VV-specific CD4 T-cell response (3). Data for the human CD4 T-cell response to cytomegalovirus, in contrast, were somewhat consistent with immunodominance. Subjects recognized a median of 12 ORFs per person (of 213 ORFs studied), with the top 6 ORFs accounting for about 40% of the overall response (4). In humans, HLA variation is expected to influence the identity of immunodominant and immunoprevalent antigens in specific individuals. Model systems have identified additional factors controlling epitope choice for CD4 T cells, including naïve T-cell repertoire (5), antigen abundance (6), antigen folding (7), protease processing and epitope-flanking regions (8), and antigenic competition (9).

Vaccinia virus is an orthopoxvirus that causes an infection that resolves completely in several weeks in immunocompetent hosts. CD4 T-cell memory persists for decades despite the absence of

antigen reexposure, but little is known about the detailed architecture of long-term memory. The monotonic decline of specific antibody levels supports lack of intermittent boosting (10). VV has over 200 ORFs, so each human has a myriad of potential CD4 reactive T-cell specificities. Herpes simplex virus 1 (HSV-1) also has a complex proteome, but in contrast to self-limited VV infections, HSV-1 infections are chronic with intermittent reactivations. Following initial epithelial replication, the virus establishes persistence in the innervating sensory ganglia. Intermittent reactivation of latent HSV-1 in essentially all HSV-1-infected persons (11) results in periodic viral antigen exposure to HSV-1-specific memory T cells (12).

To determine the breadth and persistence of CD4 T-cell immunodominance in acute and chronic human viral infections, we compared patterns of CD4 T-cell immunodominance between recent and remote VV recipients. The immune responses of persons chronically infected with HSV-1 were also investigated as an example of a chronic, intermittently reactivating infection. The immunodominance curves obtained were modeled mathematically, and specific viral proteins that were repeatedly observed to be immunodominant were also identified. We believe that the

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novel methods used in this report provide the first detailed comparisons of immunodominance in the human CD4 T-cell response to cleared and chronic large-genome viral infections.

MATERIALS AND METHODS

Participants and specimens. Adults receiving immunization with Dryvax vaccine for occupational health, U.S.-born persons with a history of childhood vaccination, and adults with HSV-1 infection (13) gave signed informed consent. The University of Washington Institutional Review Board approved all study procedures. Blood was obtained 20 to 60 months after recent VV vaccination. Age was used to estimate time since childhood VV vaccination, based on the U.S. vaccination strategy in the 1950s to 1970s to vaccinate at 3 to 12 months (14). Peripheral blood mononuclear cells (PBMC) were cryopreserved after Ficoll enrichment.

Cell and viral culture. VV NYCBH and HSV-1 E115 were cultured (13). For virus-specific CD4⁺ oligoclonal microcultures (OCM), PBMC were stimulated 20 h with UV-treated lysates of mock- and virus-infected cells. Cells were stained for CD8, CD137, and CD4 (BD), washed, and 7-amino-actinomycin D (BD) labeled. Live, CD4⁺, CD8⁻, and CD137⁺ cells were sorted (FACSARIA II, BD) and plated at 4 to 8 cells/well with 10⁵ allogeneic gamma-irradiated PBMC. Phytohemagglutinin (PHA) was used as the primary mitogen (15). This procedure has been shown to enrich and select virus-specific CD4 T cells (13). Natural human interleukin-2 (IL-2) (Hemagen) was added (32 U/ml) at 48 h. The base T-cell medium (TCM) has been described (15). Culture medium changes were done biweekly for 3 weeks. To raise bulk cultures of VV-specific CD4 T cells, we either used the CD137 staining method detailed above and sorted live, CD4⁺, CD8⁻, and CD137⁺ cells in bulk and stimulated them identically or alternatively stimulated whole PBMC with UV-treated vaccinia virus and supported proliferation with IL-2 as described previously (16).

Antigens. Viral antigens were UV-treated mock-, VV-, or HSV-1-infected cell sonicates (15). For VV, BSC40 cells were infected at a multiplicity of infection (MOI) of 0.01 and harvested at 48 h, while for HSV-1, Vero cells were infected at an MOI of 0.001 and harvested at 96 h; in both cases, monolayers showed confluent cytopathic effect by light microscopy. Peptides (Sigma, St. Louis, MO) were used at 1 µg/ml for intracellular cytokine cytometry (ICC) or cultured enzyme-linked immunospot assay (ELISPOT) or at 5 µg/ml for direct ELISPOT. The VV expression library and negative-control *Plasmodium falciparum* and *Francisella tularensis* ORFs and ORF fragments have been described (6, 13). Controls included no vector or pXT7 (6). Proteins diluted in TCM were arrayed in a 12 by 24 grid and pooled row- and column-wise to give final concentrations of each protein of 1:12,000. Single proteins were tested at 1:4,000. The HSV-1 ORFeome set (13) proteins were pooled in a 10 by 12 grid as above and used at similar concentrations. Thirteen-mer peptides overlapping by 9 amino acids (aa) covered VV ORFs WR_059 (protein E3L) (17), WR_129 (A10L), and WR_101 (H3L) (18). Peptides were arrayed in rectangular grids (E3L, 8 by 6; A10L, 24 by 12; H3L, 8 by 10) for row- and column-wise pools.

VV antigen/epitope discovery for bulk cultures. Bulk VV-specific CD4 T-cell cultures expanded from PBMC were interrogated with both genomic library-based and full-length ORF methods as described previously (16, 17). Molecular truncations were made to detect short antigenic viral polypeptides in initial hits (16, 17). Peptides (13-mer; overlap of 9 aa) at 1 µg/ml that stimulated >0.3% of bulk CD4 T cells to express gamma interferon (IFN-γ) (17) were scored as reactive.

Immunodominance proliferation assays. For the CD137-enriched CD4 T-cell OCM, sequential [³H]thymidine proliferation assays (15) were done. Autologous irradiated PBMC (3 × 10⁴ to 5 × 10⁴/well) were antigen-presenting cells (APC), [³H]thymidine was added after 36 to 60 h, and cells were harvested 6 to 18 h later. To interrogate a viral ORF matrix set, ~50% of responder OCM cells were split into 24 (HSV-1) or 38 (VV) wells of 96-well U-plates. Recombinant antigen pools and UV-mock and -virus controls were tested. When the [³H]thymidine data returned, the remaining OCM cells were plated into enough wells to match the product

of the number of positive-row and -column pools, plus two. Single antigens from intersections of positive rows/columns were tested. For one person with many OCM reactive with VV proteins E3L or H3L, residual cells were used in a final [³H]thymidine assay with matrix-arrayed peptide pools. Cells from multiple OCM reactive with VV protein A10L were combined into 2 pools prior to peptide analysis.

T-cell assays. Direct *ex vivo* 20-hour PBMC IFN-γ, IL-2, and tumor necrosis factor alpha (TNF-α) ICC for responses to whole VV included costimulatory monoclonal antibodies (MAbs) (anti-CD28 and -CD49d) and brefeldin A (17). Staining (17) used Violet live/dead and fluoro-chrome-conjugated MAbs: CD8 PerCP-Cy5.5, CD4 allophycocyanin-H7, CD3 energy-coupled dye (phycoerythrin [PE]-Texas Red), IFN-γ PE-Cy7, IL-2 PE, and TNF-α fluorescein isothiocyanate (FITC) (all BD). Gating used the lymphocyte forward/side scatter window, followed by live cells, CD3⁺ cells, and then CD4⁺ CD8⁻ cells. Net percentages of cytokine-positive live CD3⁺ CD4⁺ CD8⁻ cells were calculated by subtracting mock responses. To use *ex vivo* PBMC CD137 expression for analysis, cells were stimulated 20 h without costimulatory MAb/brefeldin A, stained with Violet live-dead and CD137 APC, CD8 PerCP-Cy5.5, and CD4 FITC, washed, fixed, and analyzed. Gating used the lymphocyte forward/side scatter window, followed by live cells and then CD3⁺ CD4⁺ CD8⁻ cells. The net percentages of live CD4⁺ CD8⁻ CD137⁺ lymphocytes were analyzed by subtracting values from mock-stimulated cells. Direct *ex vivo* IFN-γ ELISPOT (17) results are mean (of duplicate) spot-forming units (SFU)/10⁶ PBMC minus mean background. For IFN-γ ICC tests of expanded bulk responders, we incubated 3 × 10⁵ responders with an equal number of carboxyfluorescein succinimidyl ester (CFSE)-labeled autologous PBMC for 6 h with costimulatory MAb and brefeldin A. Staining used surface CD3 APC, CD4 PerCP, and intracellular IFN-γ PE. Gating used the lymphocyte forward/side scatter area followed by CD3⁺ cells and then dump-gated the CFSE⁺ cells used as APC (19).

Statistics. Immunodominance was estimated by calculating the percentages of total CD4 ORF level antigen responses for the top four viral ORFs. Quantitative immunodominance measures were modified magnitude-breadth analyses (20). For each participant, the log₁₀ cumulative number of ORF level responses was plotted versus the linear cumulative breadth, starting with the most dominant response, with both measures normalized to percentages of the total for that person. The X-Y data sets were natural log fit (Excel; Microsoft, Redmond, WA) with default trendline parameters. Reported variables are from resultant curves, $y = m[\ln(x)] - b$, and R^2 values. Integrated analyses of the data from the VV ($n = 8$) and HSV subjects ($n = 4$), considered groups, were done by summing either the number of CD4 responses per rank or the number of CD4 responses per ORF, excluding ORFs with 3 or fewer CD4 cells per person. Summed responses were plotted versus the log₁₀ of the rank. The X-Y data sets were fit to a linear model. Calculated values from curves [$y = m(x) - b$] and R^2 values were recorded.

RESULTS

CD137 upregulation identifies virus-specific CD4 T cells. We recruited persons with a history of recent ($n = 4$) or distant ($n = 4$) VV vaccination and 4 persons seropositive for HSV-1 (Table 1). A small proportion of live, CD4⁺ T cells expressed intracellular IFN-γ, TNF-α, or IL-2 after 20 h of stimulation with whole virus (Fig. 1A; Table 2). We have previously shown for both vaccinia virus and HSV that these cytokine-positive cells contain a mixture of monofunctional cells expressing one cytokine and polyfunctional cells expressing two or three of the cytokines tested (17, 23). We tested CD137 responses side by side and observed similar responses (representative data are shown in Fig. 1B). Recently, we reported that CD137 could also be used to sort and expand memory human CD4 T cells reactive with HSV-1 or recent vaccinia virus vaccination (13). To determine if CD137 could be used to enrich VV-specific CD4 T cells from

TABLE 1 Participants studied in this report

Participant ID ^a	VV vaccination history ^b	HSV-1/HSV-2 serostatus ^c	HLA class II type ^d
V3	2, 30 mo	NA ^e	DRB1*15, *03, DQB1*0201, *0602
V8	2, 20 mo	NA	DRB1*1201, *0405, DQB1*0301, *0401
V9	2, 44 mo	NA	DRB1*1501, *0401/33, DQB1*0301, *0602
V12	1, 60 mo	NA	DRB1*11:01, *11:04, DQB1*03:01
V16	1, 49 yr	NA	DRB1*11:01, *15:02, DQB1*03:01, *06:01
V17	1, 41 yr	NA	ND ^f
V18	1, 57 yr	NA	DRB1*04:04, *15:01, DQB1*03:02, *06:02
V19	1, 46 yr	NA	DRB1*04:01, *07:01, DQB1*02:02, 03:02
H1	NA	Pos/neg	DRB1*0701, DQB1*0202
H5	NA	Pos/pos	DRB1*0101, *0301, DQB1*0201, *0501
H6	NA	Pos/pos	DRB1*1104, *1401, DQB1*0301, *0503
H15	NA	Pos/neg	DRB1*07:01, *12:01, DQB1*03:01, 03:03

^a V refers to VV with participants V3, V8, V12, and V9 from previous reports (6, 16, 21), with V16 through V19 new to this report. H refers to HSV-1 with persons H6, H5, and H1 from previous reports (13) and H15 new to this report.

^b Number of lifetime VV vaccinations and interval in months between the most recent vaccination and phlebotomy (V3 through V12) or chronologic age at phlebotomy (V16 through V19).

^c Pos and neg, presence and absence, respectively, of type-specific IgG by immunoblot assay (13).

^d HLA types reflect the nomenclature current at the time the typing was performed. An explanation and reconciliation tools are available (22).

^e NA, not applicable.

^f ND, not done.

persons several decades after vaccination, we repeated bulk level CD4 T-cell analyses using the exact same method. Again, VV-reactive CD4 cells in the CD137^{high} origin cultures were considerably enriched, with the expanded, bulk, T-cell lines showing large IFN- γ responses to whole VV antigen (representative data are shown in Fig. 1C).

Assessing CD4 T-cell dominance and diversity for VV and HSV-1 antigen recognition at the functionally clonal level. To assess immunodominance at the single-cell level, we then modified this method for both recent and distant vaccinees by sorting CD137^{high} immediately after a short, 18-hour *ex vivo* stimulation into near-clonal cultures. Functionally clonal responses within these microcultures were then dissected via multiplexed fine-specificity analyses using a complete protein set. We distributed CD3⁺ CD4⁺ CD137^{high} cells immediately after sorting into microwells at 4 to 8 cells/well and stimulated the cells with a nonspecific mitogen, PHA (24). The resultant progeny of the few sorted

PBMC/well were termed oligoclonal microcultures (OCM). These cells reliably expanded to several hundred thousand daughter cells by day 14, at which time readout assays were initiated. OCM cells remained viable and antigen reactive through day 23, permitting initial, confirmatory, and downstream assays without the need for any additional exposure to a mitogen.

The specificities of the oligoclonal populations in the OCM wells were obtained using two sequential assays. We first interrogated 60 to 72 OCM per person against the entire respective virus proteome set, represented as protein pools from rows and columns in a matrix. It was quite infrequent to observe a positive response to whole-virus lysate in the first assay but not to observe at least one positive-row pool and -column pool of individual viral proteins. Less than 10% of virus-reactive OCM for the set of 12 subjects studied had this pattern. Intersection(s) of reactive row(s) and column(s) each identified a candidate reactive viral ORF (Fig. 2A). To confirm antigen specificity, each candidate was tested in the second assay in a subset of 21 to 60 OCM per person. The criteria for selecting OCM for the secondary assay were a positive response to whole-virus lysate, at least one column and row pool in the first assay, and the physical presence of enough healthy cells as assessed by light microscopy to permit an estimated 5,000 cells per well to be added to the second assay. We observed large signal-to-noise ratios at both assay stages (Fig. 2B and C). Positive-control whole-virus responses were brisk. Among the 12 persons, the median number of reactive ORFs per OCM was 2 (average \pm standard deviation, 2.2 ± 1.15 ; range, 1 to 6). The OCM were therefore proven to be monospecific to oligonucleotide specific for virus-reactive CD4 T cells.

Immunodominance architecture of the virus-specific CD4 T-cell response. We studied 21 to 60 virus-reactive OCM per person in the initial assay, yielding fine-specificity data for 37 to 153 virus-specific CD4 T cells per participant in the second assay (Table 2). On an integrated basis, we obtained 1,134 unique antigen level hits for virus-specific CD4 T cells among the 12 study subjects, on average determining the ORF level specificities of 94 virus-specific CD4 T cells per person. The positive responses were displayed as histograms with each bar representing a viral ORF and ordered from most- to least-abundant responses. Generally, pronounced immunodominance was displayed by 2 to 4 viral ORFs per participant (Fig. 3). Several proteins were then recognized in a subdominant fashion, followed by a long tail of viral

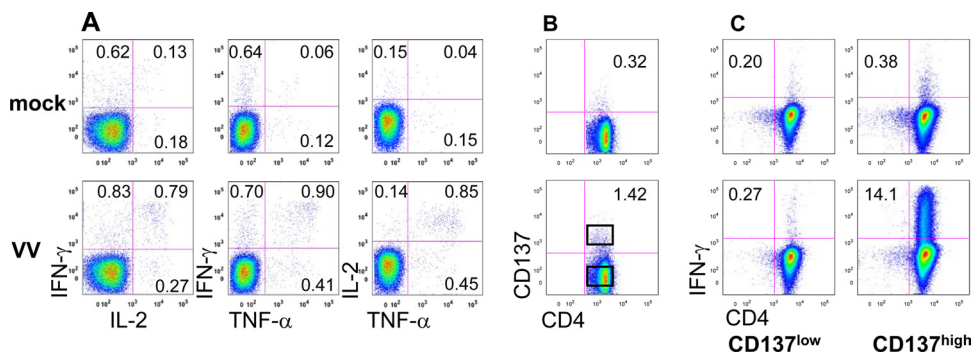


FIG 1 CD137 upregulation identifies virus-specific CD4⁺ T cells. Direct *ex vivo* PBMC expression profiles of cytokines (A) and CD137 (B) from remote VV vaccinee V16 to mock and vaccinia virus (VV) antigens. Percentages of positive cells, gated on live CD3⁺ CD8[−] CD4⁺ cells, are provided. (B) Rectangles are gates for sorting CD137^{low} or CD137^{high} CD4 T cells. (C) IFN- γ expression of bulk cultures of CD137^{low}- or CD137^{high}-sorted CD4 T cells tested to mock and VV antigens using autologous APC. Numbers represent the percentages of cells in the upper right quadrant.

TABLE 2 Characteristics of OCM and CD4 breadth and immunodominance data derived from CD137^{high} PBMC

Participant ID ^a					<i>In vitro</i> -expanded cells, % CD4 IFN-γ ⁺				OCM studied ^b	ORF level hits ^c	CD4 breadth ^d	% Top 4 ORF dominance ^e
	Direct <i>ex vivo</i> % of live CD4s											
					CD137 ^{high} origin		CD137 ^{low} origin					
	CD137	IFN-γ	IL-2	TNF-α	Mock	Virus	Mock	Virus				
V3	1.09	0.41	0.25	0.29	ND ^f	ND	ND	ND	23	71	33	28
V8	0.42	0.07	0.07	0.18	ND	ND	ND	ND	60	84	25	39
V9	1.18	0.27	0.22	0.35	ND	ND	ND	ND	48	117	44	35
V12	1.1	0.87	0.76	1.13	ND	ND	ND	ND	52	153	48	64
V16	0.15	ND	ND	ND	0.38	14.1	0.2	0.27	52	105	38	32
V17	0.12	ND	ND	ND	0.30	5.25	0.149	0.153	54	95	44	39
V18	0.02	ND	ND	ND	0.16	1.91	0.08	0.06	38	116	38	42
V19	0.06	ND	ND	ND	0.45	31.5	0.21	0.28	55	95	38	33
H1	0.17	ND	ND	ND	ND	ND	ND	ND	21	37	20	49
H5	0.24	ND	ND	ND	0.18	15.14	0.13	0.52	34	113	42	32
H6	0.53	ND	ND	ND	0.22	19.1	0.19	0.75	45	113	35	44
H15	0.06	ND	ND	ND	ND	ND	ND	ND	35	75	32	30

^a See Table 1 for participant and viral infection data.
^b Number of OCMs with positive row and column pool level responses to viral antigens used for assays with single viral proteins.
^c Number of positive reactions between individual OCMs and single viral proteins.
^d Number of unique viral ORFs recognized by the integrated group of OCM.
^e Percentage of ORF level hits triggered by the four most frequently recognized single viral proteins.
^f ND, not done.

proteins recognized by a single CD4 T-cell. The top 4 viral ORFs per person drove the responses of 28 to 64% of CD4 T cells within a person.

The pattern of reactivity was independent of the virus assayed or time since VV vaccination (Fig. 3). The cumulative immunodominance distributions were remarkably similar in shape. Each breadth-dominance curve closely fit a logarithmic equation of the form $y = [m(\ln) - b]$ with R^2 values near 1.0. The equation parameters, particularly the “ m ” term describing the steepness of the curve, were similar regardless of the duration of CD4 memory for VV and for VV in comparison to HSV-1. This indicates a similar proportional drop-off in abundance with decrease in rank for both HSV-1 and VV and regardless of time since infection for VV.

Next, we summed the total number of CD4 T cells within each rank for all participants by group (VV or HSV-1) and plotted the log of this sum versus the log rank (Fig. 4). These plots approximated linearity for the 8 VV participants ($R^2 = 0.96$) and 4 HSV-1 participants ($R^2 = 0.95$) and fit Zipfian distributions (25) with slopes equaling -0.7 and -0.8 , respectively. This Zipf distribution suggests that the relative abundance of CD4 T cells reactive with a particular ORF can be predicted based entirely on its rank and the slope of the log-log plot (Fig. 4A). The plots of recent and distant VV recipients were superimposed, implying equivalent rank-abundance traits between groups (Fig. 4B).

Breadth of the virus-specific CD4 response. The CD4 T-cell breadth per person ranged from 25 to 48 ORFs in recent vaccinees and from 38 to 44 ORFs in distant vaccinees. For HSV-1, the CD4 T-cell breadth ranged from 20 to 42 HSV-1 ORFs. Each person recognized 11% to 22% of the VV ORFs tested. In comparison, for HSV-1, participants recognized a higher percentage of the HSV-1 ORFs, ranging from 27% to 57% of the 74 HSV-1 ORFs studied.

CD4 T cells may recognize one or more peptides within a protein. To investigate whether responses that were dominant at the ORF level also showed immunodominance at the peptide level, we

tested overlapping peptides from three VV proteins that showed immunodominance. OCM from remote VV subject V17, reactive with A10L (WR_129) or E3L (WR_059) were decoded to the 13-mer peptide level, as were OCM from remote VV subject V18, reactive with H3L (WR_101). For E3L, with 48 peptides tested, 10 of 10 OCM reacted with E3L peptides 49 to 61 (KALYDLQRSAM VY). For H3L, 80 peptides were tested, and the data were consistent with the recognition of two overlapping 13-mers, H3L 221 to 233 (NEMKINRQILDNA) and H3L 225 to 237 (INRQILDNA KYV) by each of 11 distinct OCM. This implies recognition of one epitope approximated by INRQILDNA (H3L 225 to 233). Similarly, two pools, made from cells from 10 or 8 OCM reactive with full-length A10L, each reacted with only one peptide, A10L 73 to 85 (GINYLIDTTSREL), among the 288 13-mers tested. Overall, a single epitope was internally dominant within each immunodominant ORF tested.

CD4 immunodominance directly *ex vivo*. Separately, we used published (16, 17) methods to define VV CD4 T-cell antigens, and then epitopes, for participant V9. In preliminary work, a VV-reactive bulk CD4 T-cell line from this subject recognized 23 VV polypeptides (not shown). Overlapping peptides were obtained for these ORFs, and at least one reactive peptide was found per ORF when queried with the bulk CD4 T-cell line using IFN-γ readouts (not shown). We then examined direct *ex vivo* PBMC responses to these CD4 antigenic peptides. We observed that responses to peptides in 9 of these ORFs were detectable at ≥ 5 SFU/10⁶ PBMC with an IFN-γ ELISPOT readout. When arranged by magnitude, the peptide immunodominance pattern (Fig. 5) was similar to that observed for vaccinia virus ORFs using the indirect CD137 method.

Characteristics of immunodominant viral proteins. Several of the VV and HSV-1 proteins with in-person immunodominance (Table 3) were long polypeptides that were also abundant in purified virions (26, 27). Four of the nine most-abundant VV virion proteins, encoded by WR_129, WR_122, WR_148, and

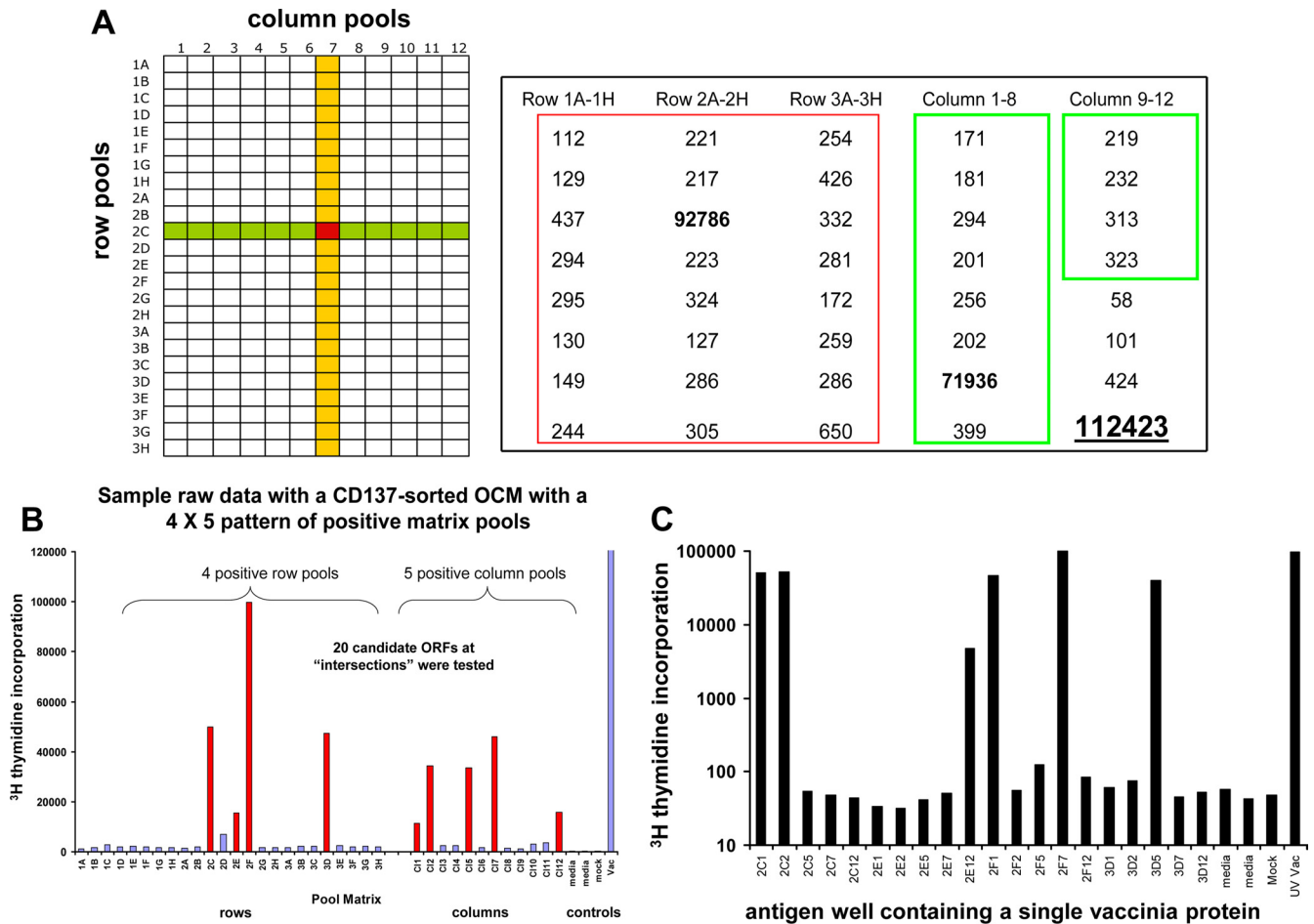


FIG 2 Schema and representative raw data for oligoclonal microcultures. (A) Method of arraying VV polypeptides into 12 rows and 24 columns. Some pools contained negative controls (left). Sample [^3H]thymidine proliferation data from one OCM are shown on the right. Row 2C and column 7 are positive. Confirmatory proliferation data using just the VV antigen at the row 2C-column 7 intersection, shown in red, were positive (not shown). Whole-UV-VV control at lower right. (B) Representative row and column [^3H]thymidine proliferation data from an OCM with a complex pattern of reactivity, showing 4 positive-row and 5 positive-column pools. There are 20 candidate antigenic VV proteins at the intersections. Positive-control UV-VV at right. (C) Each candidate antigenic polypeptide was assayed by [^3H]thymidine proliferation. There were six hits. The row 2C pool contained two positive antigens, 2C1 and 2C2.

WR_101, were immunodominant in at least two persons. Similarly, the envelope HSV-1 glycoproteins gB (encoded by gene *UL27*) and gD (encoded by *US6*), similar in sequence to those of HSV-2 vaccine candidates (28), were frequently immunodominant. The HSV-1 immediate early regulatory protein ICP4 (*RS1*) is also abundant in virions (27) and was dominant in each of 4 subjects. There were some exceptions to this pattern, as occasionally short, nonstructural proteins also displayed immunodominance. For example, the VV protein encoded by WR_059 (E3L), which was immunodominant in four persons, is only 190 aa long and may be absent from virions (26, 29). The VV protein encoded by WR_118 (D13L), dominant in 5 persons and prevalent in 8, is present in trace amounts in virions. Overall, there were 9 VV proteins detected as reactive in at least 75% of the subjects regardless of rank, encoded by the following genes: WR_118 (8 persons), WR_072 and WR_122 (7 persons), and WR_052, WR_070, WR_091, WR_098, WR_101, and WR_129 (6 persons each). WR_118 is also known as the target for rifampin resistance. WR_072 encodes a single-stranded DNA-binding phosphoprotein that is also known as I3L. Among the remaining frequently immuno-

genic vaccinia virus proteins, WR_070 (I1L) and WR_091 (L4R) are both DNA-binding proteins, WR_098 (J6R) is a DNA-dependent RNA polymerase, WR_052 (F13L) and WR_101 (H3L) are envelope proteins, and WR_129 (A10L) is a core protein. The HSV-1 UL40 and US12 proteins, undetectable in virions, could show immunodominance; the latter is quite short. We also noted that identical HSV-1 and VV ORFs could be immunodominant for CD4 responses in persons who did not share HLA class II loci (Tables 1 and 3).

DISCUSSION

We report proteome-wide examination of the fine architecture of human CD4 T-cell immunodominance for two complex microbes. Contrary to VV CD4 experiments in mice (3), we found pronounced immunodominance in recent VV vaccinees. Surprisingly, broad tails of subdominant and low-abundance responses were maintained in remote VV vaccinees, such that the architecture of the CD4 T-cell dominance curves were mathematically indistinguishable in recent and remote vaccinees. We conclude that a single antigen exposure to VV primes a diverse CD4 mem-

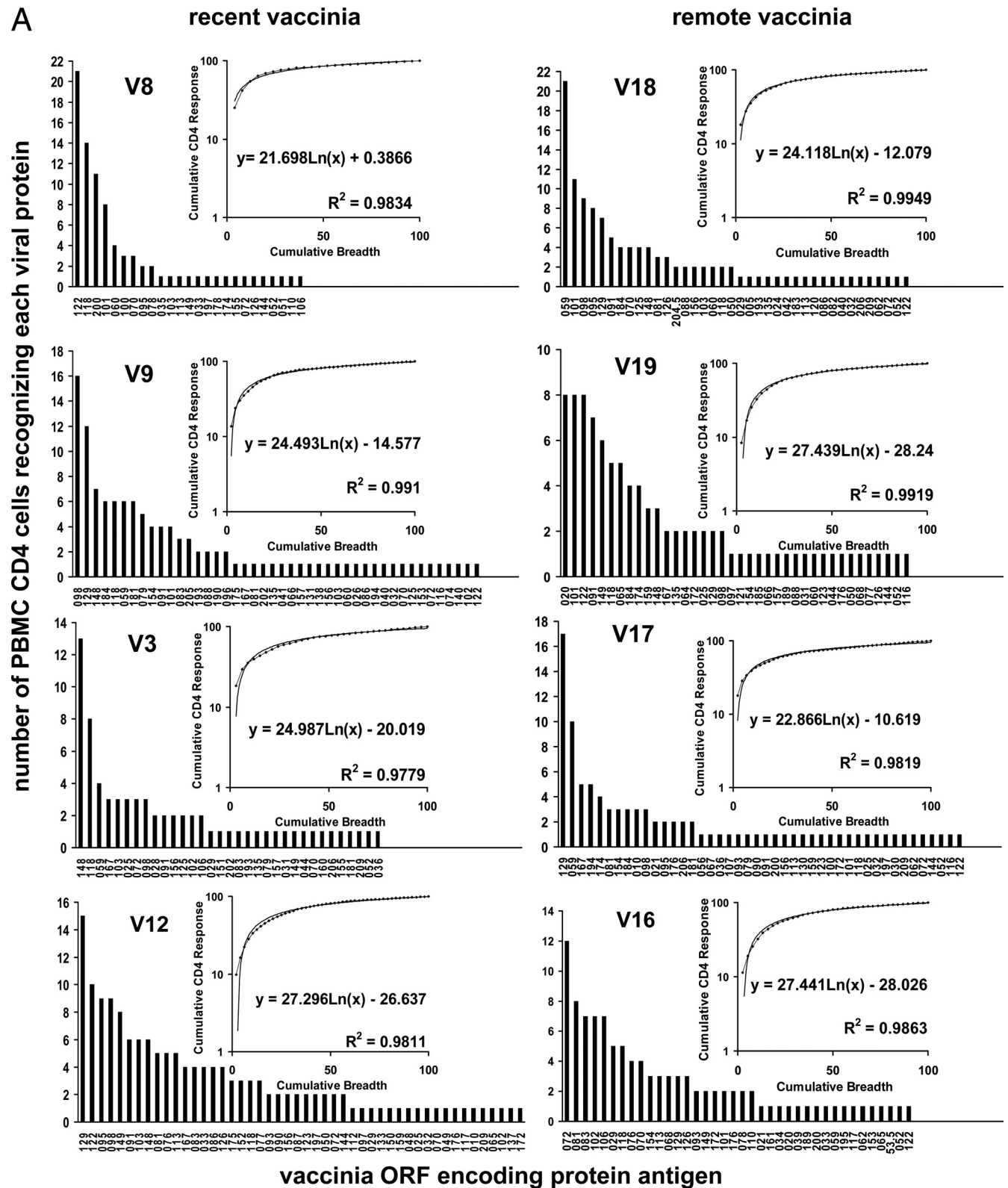


FIG 3 CD4 T-cell reactivity to VV (A) and HSV-1 (B) proteins. Histograms represent one person each with recent (“recent vaccinia”) or remote (“remote vaccinia”) VV vaccination (V12 and V16) (A) or HSV-1 infection (B). The y axes carry the numbers of separate oligoclonal microcultures (OCM) cultured from CD137^{high}-sorted virus-specific PBMC-derived CD4 T cells reacting with each viral protein. The x axes carry the names or numbers of individual viral ORFs using standard nomenclature. Viral proteins are ordered from most to least dominant. Insets are breadth-dominance curves from each histogram. The x and y axes are the cumulative percentages of the CD4 T-cell breadth and the ORF level hits, respectively. Trend line natural log fits display parameters and R^2 with experimental data.

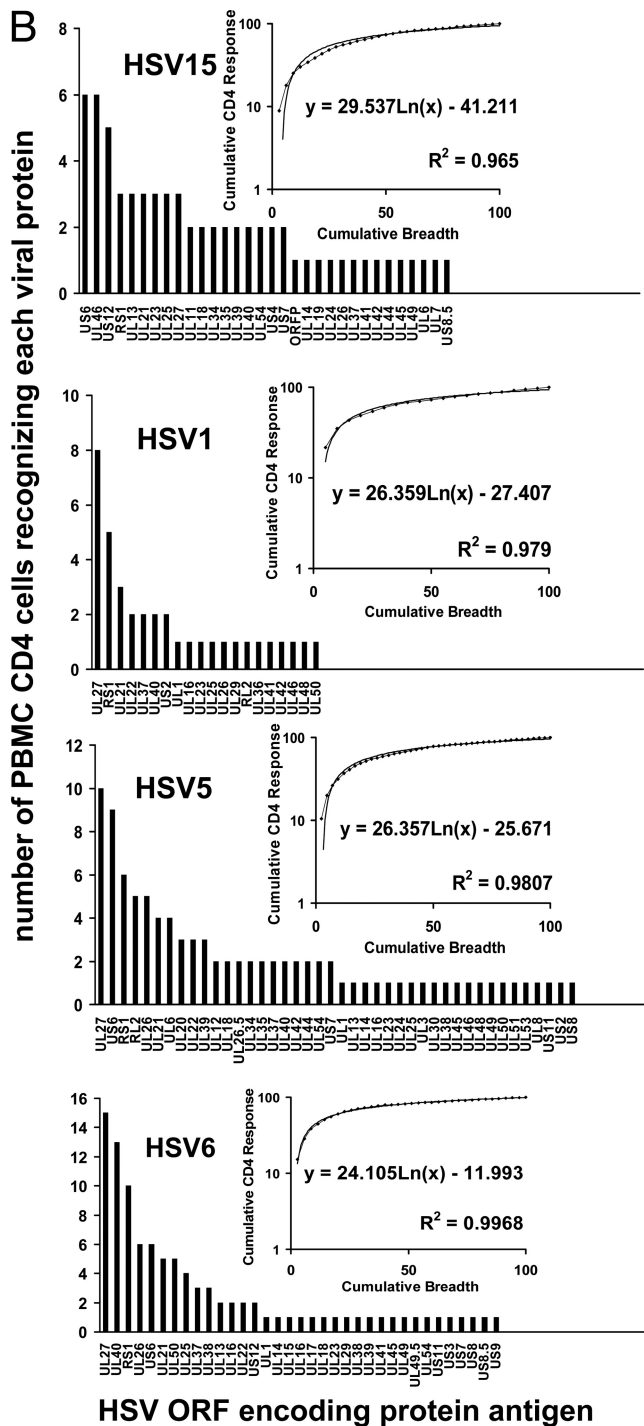


FIG 3 (Continued)

ory that persists for decades. A similar immunodominance pattern was noted for HSV-1-specific CD4 T cells, despite the very different pathogenesis of HSV infections. Periodic reexposure to endogenous HSV-1 antigens after the infection is established does not seem to fundamentally influence the nature of CD4 T-cell immunodominance.

There are several novel aspects of this report. CD4 T-cell antigens and epitopes have previously been reported for both VV and

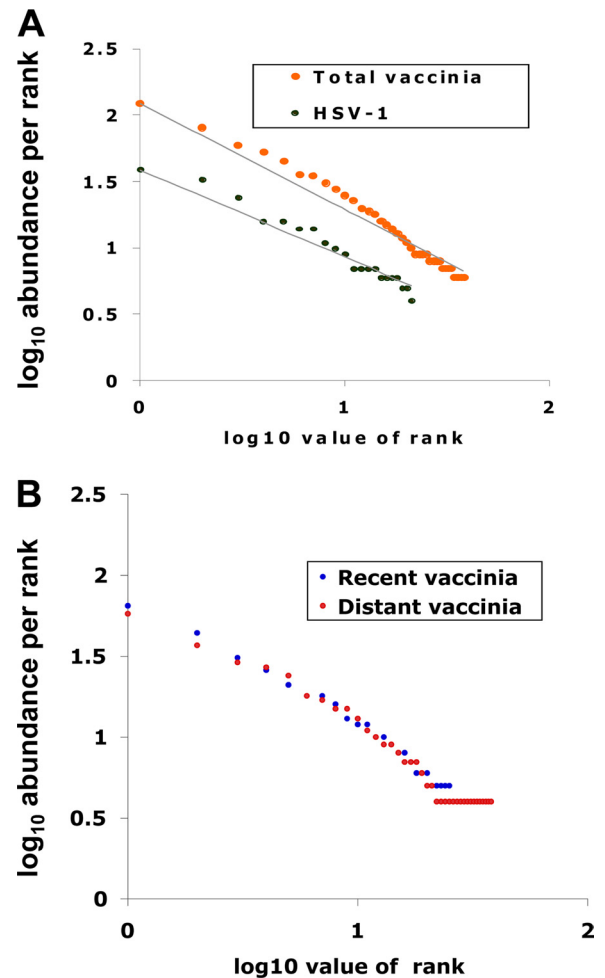


FIG 4 (A) Abundance rank plots of log abundance per rank versus log rank in eight vaccinia virus recipients and four HSV-1-infected persons. The x axis represents the log values of rank; the y axis represents the log values of abundance per rank in each group. (B) Abundance rank plots of log abundance per rank versus log rank in four recent and four distant vaccinia virus recipients; the x axis represents the log value of rank; the y axis represents the log value of abundance per rank in each group. Gray lines represent the linear fit to the data with the y -intercept equalized to the data. Reactive antigens with an abundance of 3 or fewer were not included in the plots.

HSV-1 (13, 30–33), but their immunodominance has not been rigorously addressed in humans. We are aware of one study that measured human CD4 T-cell responses to VV proteins directly *ex vivo*, but the protein space investigated covered only 4 VV membrane proteins (34). ELISPOT for IFN- γ , the assay used in that report, may fail to detect low-abundance or functionally exhausted responses. In contrast, we investigated the entire proteomes of two microbes. Earlier CD4 T-cell studies of VV and HSV-1 (6, 13, 16, 17) used fundamentally different responder cell populations, namely, highly polyclonal cell lines. These responder cells were suitable for scoring individual proteins as reactive or nonreactive but not appropriate for counting individual virus-specific CD4 T cells. In this report, we interrogate responder cell cultures that are conceptually clonal, using combinatorial methods to detect reactivity with near-complete viral proteome sets. This allows actual determination of the number of CD4 T cells reactive with each viral ORF and thus precise detection of immu-

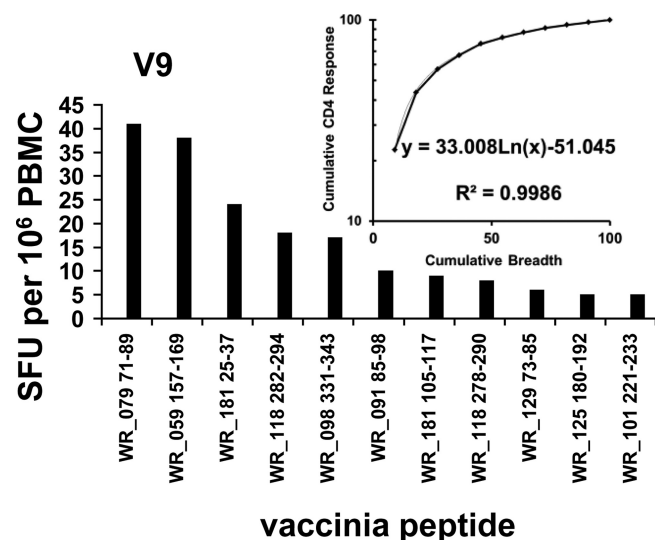


FIG 5 Immunodominance architecture of VV-specific CD4 responses in participant V9 measured by *ex vivo* IFN- γ ELISPOT. Bar labels are names of VV WR ORFs and amino acid numbers of peptides. The inset shows the breadth-dominance curves corresponding to the histogram. The x axis represents the cumulative percentage of CD4 breadth. The y axis represents the cumulative percentage of CD4 ORF level hits. Peptides are ordered as in the histogram from most to least dominant. The trend line is a natural log fit curve with parameters and R^2 with experimental data displayed.

nodominant antigens and dominance patterns in circulating CD4 T cells.

In quantitative analysis, we were not able to distinguish the T-cell CD4 immunodominance architecture between recent and past VV vaccinees. Both groups of persons had dominant responses to a few antigens and a wide set of subdominant and minority responses. VV ORF-reactive CD4 T cells were rank ordered per Zipf's law, a distribution that assumes that the abundance of T cells specific to each antigen is a mathematical function of antigen rank within each person. The similarity of the slopes of the rank dominance curves in Fig. 4 implies that the architecture of the immunodominance hierarchy is the same for both recent and distant VV infections and for VV and HSV-1 infections. The reason for this precise structure of antigen specificity among CD4 T cells is unknown. Equal priming of T cells reactive with many antigens, followed by differential rates of CD4 T-cell decay, is one possibility. This seems unlikely, because over decades following VV exposure, the rather broad tails of CD4 T-cell responses that we observed would be expected to narrow, unless the rates of clonotype decay themselves were each dynamic and slowed over time as T cells with defined specificities became rarer. Stochastic effects could be important if chance events during the first contact between a naïve T cell and APC predict proliferation and survival. If these initial contacts were commoner for some antigens than others, this stochastic theory could be reconciled with our observation that the same VV antigens tend to be immunodominant in multiple persons. A higher number of initial APC-naïve cell contacts would lead to a higher probability of advantageous priming events. We previously observed that immunoprevalent VV CD4 T-cell antigens tended to be abundant virion envelope and capsid proteins (6, 16, 17), which might be expected to give rise to abundant peptide-HLA complexes on APC.

In some model systems, APC process or retain certain HLA-peptide complexes more efficiently expressed than others (35). This competition presumably results in a selective advantage for early expansion and higher persistent levels of T cells specific for these antigens. While protein abundance could be one factor feeding into the number of HLA-peptide complexes, we noted examples for both VV and HSV-1 in which viral proteins that were of very low abundance or undetectable in virions were nonetheless immunodominant, often in several people (Table 3). A mathematical model based on murine influenza data predicted that differential antigen expression explains CD8 T-cell immunodominance after a secondary exposure to virus but not following primary infection (36). Our results revealed that CD4 T-cell immunodominance established after a single primary antigenic exposure to VV is partially but not completely explained by antigen abundance.

Naïve T-cell clonotype abundance seems to be predictive of immunodominance in inbred mice (37) and may also operate on an HLA allele-specific basis in humans (38), albeit there are examples of inverse relationships between naïve and memory abundance for CD4 T cells (39). This factor alone would not explain our data indicating that the same antigens can be dominant in subjects who do not share HLA alleles. Our subjects were quite diverse for HLA class II alleles and therefore would be expected to have diverse naïve T-cell repertoires. To address the issues of HLA type and the naïve repertoire, we would need to determine virus-specific fine specificity to the levels of exact peptides, HLA restriction, and even T-cell clonotypes and compare memory to naïve T cells.

It is clear from our data that memory CD4 T-cell responses to VV are extraordinarily long-lived. The contributors to memory T-cell survival in the absence of antigen are incompletely understood. Memory CD4 T cells can express receptors for the survival/proliferation cytokines IL-7 and IL-15 (40). Intercurrent infections seem to perturb CD4 less than CD8 memory (41), but it is possible that coinfections such as Epstein-Barr virus (EBV) or influenza virus infections could influence dominance via heterologous immunity (42).

Limitations of our study include the number of persons studied, potential bias introduction by CD137 selection or proliferation assays, and impurities and small gaps in the viral proteome sets. Each of the four HSV-1 persons studied recognized UL27 and RS1, but if we had studied more persons, exceptions to this high population prevalence might emerge. Each of the 8 VV-vaccinated persons had a similar CD4 T-cell immunodominance architecture, although variation in dominance patterns and in the identity of dominant antigens would be likely in a larger population. There are no data indicating that any T-cell subsets fail to upregulate CD137 after TCR ligation and would thus be excluded from our data, albeit this is possible. Because our restimulation cultures contained only 4 to 8 seed cells, cell competition during the expansion phase was minimized. We very frequently detected several specificities from each microculture well, indicating that attrition due to failure to proliferate was quite uncommon among the sorted CD137^{high} cells. Memory CD4 T cells exist in several subsets, with effector-memory and effector cells having less proliferative potential (43). MAb ligation of CD137 is strongly costimulatory for cell subsets thought to be functionally exhausted (44, 45). Future experiments with sorted CD4 T-cell responder subsets should be able to address this hypothesis.

TABLE 3 VV and HSV-1 proteins showing within-person immunodominance

Virus ORF ^b	Participant ID ^a											
	VV recent				VV remote				HSV-1			
	V3	V8	V9	V12	V16	V17	V18	V19	H1 ^c	H5	H6	H15 ^c
VV												
WR_118	x	x	x		x			X				
WR_059	x		x			x	x					
WR_098	x		x	x			x					
WR_101		x					x	x				
WR_129			x	x		x						
WR_072	x				x							
WR_095				x			x					
WR_122		x		x				x				
WR_148	x		x									
WR_020								x				
WR_025	x											
WR_065								x				
WR_029					x							
WR_081					x							
WR_083					x							
WR_091								x				
WR_102					x							
WR_103	x											
WR_106					x							
WR_149				x				x				
WR_167	x											
WR_167						x						
WR_174						x						
WR_181			x									
WR_184			x									
WR_194						x						
WR_200		x										
HSV-1												
UL27									x	x	x	x
RS1									x	x	x	x
US6										x	x	x
UL21									x			x
UL26										x	x	
UL13												x
UL22									x			
UL23												x
UL25												x
UL40											x	
UL46												x
US12												x
RL2										x		

^a See Table 1 for detailed participant and viral infection or vaccination information.

^b Identity of viral ORFs, using VV strain WR or HSV-1 strain 17 nomenclature, encoding proteins scoring in the top first to fourth ranks for each person, in rank order. Proteins recognized by an equal number of CD4 clonotypes were each included.

^c Participants H1 and H15 had flat breadth-dominance curves, and only the top three ranks are included.

It is possible that we detected progeny cells resulting from *in vitro* priming rather than memory recall. Arguing against this, *in vitro* priming typically requires specialized APC and repeated cycles of antigen exposure and leads to very low responder cell frequencies (46). We have yet to detect specific CD137 expression by CD4 T cells from persons who were seronegative for vaccinia virus, so use of this method to probe the naïve repertoire is currently out of reach. We have previously shown that a minority of HSV-1/HSV-2 doubly seronegative persons, highly exposed to HSV-1, had direct *ex vivo* cytokine and CD137 responses to HSV-1 (13).

The reason for this reactivity is unclear and could represent sensitization to noninfectious virus via interpersonal contact or cross-reactivity with other herpesviruses. We also observed cytokine reactivity to HSV peptides at relatively high levels directly *ex vivo* in a small number of HSV-1/HSV-2 doubly seronegative persons who were sexual partners of HSV-2-infected persons (47). The direct *ex vivo* nature of these observations in overnight assays is not consistent with *in vitro* priming as a cause of the positive T-cell responses in these cases for seronegative persons. For vaccinia virus, we have never observed a positive cytokine response

directly *ex vivo* (16). Because the CD137 purification method in this report also sorts cells immediately after direct *ex vivo* stimulation, we think it is unlikely that responses would be induced by *in vitro* priming. It is also possible that CD137 and the cytokine assays detect different cells. For example, there could be some degree of bystander activation for either the CD137 or cytokine readouts. Further research on these points is needed.

The viral lysates used for the initial PBMC restimulation do not appear to be a source of bias with regard to incomplete expression of the viral proteomes, as reflected by the detection of responses to both structural and nonstructural proteins for both viruses. Similarly broad recognition of structural and nonstructural proteins was noted in earlier CD4 research using the same lysate protocols (6, 13). We did note that up to 10% of OCM that recognized whole-virus lysate did not react with our recombinant protein pools, such that some of the viral proteins may have been present in low or poorly immunogenic forms in these pools, or possibly that cryptic ORFs or epitopes requiring posttranslational modifications were recognized by rare CD4 T cells in PBMC. The HSV-1 protein set used is not quite complete (13), and we have previously documented the presence of cryptic VV ORFs in our CD8 work (21). It is possible that the same T-cell clonotype could be recognizing more than one viral protein and thus give two positive ORF level readouts rather than one, but CD4 T cells typically require near identity at amino acids 1, 4, 6, and 9 to bind to HLA class II and also similar amino acids to face up to the T-cell receptor (48), such that this is quite unlikely, especially because the HSV-1 and VV genomes tend not to contain families of highly sequence-related proteins. Finally, we do not yet know how reproducible our immunodominance hierarchies would be if assayed multiple times on the same PBMC specimen. The identity of the dominant within-person ORFs would be less likely to show assay-to-assay variation than would the detection of the very subdominant ORFs detected only once each, due to sampling of rare cells in PBMC.

The viral proteins used in this report were prepared using high-throughput methods suitable for complex pathogens. Because the cells are interrogated in near-clonal conditions, some variation in the individual viral proteins may be tolerable without altering the dichotomous, yes/no readouts for each T-cell microculture. Our digital data set scored >1,000 near-clonal CD4 T-cell populations as reactive with single antigens in assays with very high signal-to-noise ratios. Protein purity is thus less essential than in analog assays performed with polyclonal responders occurring at low abundance in a background of bystander cells, such as direct PBMC assays. In contrast to many reports, we made proteins *in vitro* rather than within *Escherichia coli* or other host cells, reducing contamination with microbial innate immunity agonists and bystander proteins. The vaccinia virus and HSV-1 protein sets are not yet pure enough for direct *ex vivo* studies, a future goal. We did perform limited direct *ex vivo* work and found an immunodominance hierarchy similar to those observed with the OCM method. In subject V9, studied by both methods, there was not a strict correlation between the dominance of ORF98 at the whole ORF level and rank dominance of one constituent peptide, but this may be due to the large size of this ORF (1,286 amino acids) and the presence of additional peptide epitopes within this protein.

In conclusion, the human memory CD4 T-cell responses, both shortly and decades after infection with the completely cleared agent VV and in response to the chronic pathogen HSV-1, have mathematically near-indistinguishable patterns of immunodomi-

nance. This implies extremely efficient maintenance of even low-abundance T cells in the absence of antigen. The CD4 T-cell response invariably resembles the better-studied CD8 T-cell compartment, dominated by a few antigens that also tend to be immunoprevalent and are thus rational vaccine candidates. The net pattern of CD4 T-cell immunodominance appears to be an intrinsic property of this complex biological system.

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